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(57) Abstract

This invention relates to an isolated and purified serine/threonine kinase which is an integrin-linked kinase, designated "ILK". ILK can be used to modulate cell growth, modulate cell adhesion, modulate cell migration and modulate cell invasion. Inhibitors of ILK activity include (1) screens aimed at DNA, RNA or structural components of ILK, (2) pseudo-substrate inhibitors, such as a peptide which mimics a substrate sequence for ILK, and (3) drugs which specifically inhibit ILK activity. The diseases treated by these inhibitors include cancer, leukemia, solid tumors, chronic inflammatory disease, arthritis, osteoporosis and cardiovascular disease. Diagnostics of ILK activity include DNA-based reagents derived from the nucleotide sequence of ILK and antibodies against ILK screen biopsy-derived samples of amplified ILK DNA, or increased expression of ILK mRNA or protein. Assays which screen drugs which specifically inhibit ILK activity are included within this invention.

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Integrin-Linked Kinase, Its Inhibitors and Methods of Medical Treatment Using These Inhibitors, Gene Therapy and Pseudo-Substrate Inhibitors

Background

Proteins of the extracellular matrix (ECM) act to influence fundamental cell and tissue behaviours. ECM regulates cell structure, growth, survival, differentiation, motility and, at the organismal level, proper development. ECM proteins interact with cells via a class of cell membrane-spanning receptors called integrins. ECM is a biological signal, and the integrin receptor is a specific transducer (i.e. across the cell's plasma membrane) of this signal. Integrins are also important in proliferative disorders, mediating such processes as wound healing and inflammation, angiogenesis, as well as tumour migration and invasion.

A major biochemical response to ECM-integrin interactions is elevation of an enzymatic activity known as protein phosphorylation. Phosphorylation is important in signal transduction mediated by receptors for extracellular biological signals such as growth factors or hormones. For example, many cancer causing genes (oncogenes) are protein kinases, enzymes which catalyze protein phosphorylation reactions, or are specifically regulated by phosphorylation. In addition, a kinase can have its activity regulated by one or more distinct protein kinases, resulting in specific signaling cascades.

Research on signal transduction over the years has clearly established the importance of direct, protein-protein interactions in the cytoplasm as a major mechanism underlying the specification of signaling pathways. These interactions can, in part, be those between a receptor and a cytoplasmic protein kinase, or between a protein kinase and its substrate molecule(s).

A number of known protein kinases, such as mitogen-activated kinase (MAPK), focal adhesion kinase (FAK), and protein kinase C (PKC) have their kinase activity stimulated by integrin-ECM interaction, although no cellular protein kinase has been identified to date, which has been demonstrated to bind to an integrin molecule under physiological conditions. As such is the case, the direct molecular connection between integrins and the ECM-induced phosphorylation of cellular proteins is unclear.

As such is the case, if the direct molecular connection between integrins and the ECM-induced phosphorylation of cellular proteins were determined, products which modulated that connection would be useful therapeutics. These products could be used to modulate cell growth, cell adhesion, cell migration and cell invasion. If it were determined that a specific kinase regulates integrin function, products that regulate (for example, inhibit) the activity of that kinase could be used for the treatment of cancer, leukemia, solid tumors, chronic inflammatory disease, arthritis and osteoporosis, among other indications.

Summary of Invention

This invention relates to an isolated and purified serine/threonine kinase which is an integrin-linked kinase, designated "ILK" in this application. ILK binds to the cytoplasmic portion of the β_1 integrin molecule in a living cell, providing the first physiological evidence for interaction between an integrin and a protein kinase.

ILK can be used to modulate cell growth, modulate cell adhesion, modulate cell migration and modulate cell invasion. An amino acid sequence of ILK and an isolated nucleotide molecule encoding the amino acid sequence are part of this invention. The molecule could be cDNA, sense DNA, anti-sense DNA, single DNA, double stranded DNA. mRNA of integrin-linked kinase is part of this invention. The molecule could be a nucleotide molecule encoding an impaired amino acid sequence of a serine/threonine kinase.

Inhibitors of ILK activity are part of this invention. Inhibitors include (1) screens aimed at DNA, RNA or ILK structural components e.g. antisense ILK (i.e. synthetic DNA oligonucleotide comprising the complementary nucleotide sequence of the ILK coding region, designed to specifically target the ILK mRNA complement), (2) pseudo-substrate inhibitors, such as a peptide which mimics a substrate sequence for ILK, and (3) drugs which specifically inhibit ILK activity. These drugs may be directed at either the kinase or ankyrin repeat domains. An inhibitor may include an antibiotic, a natural or mimetic substrate for the integrin-linked kinase, and a first nucleotide molecule which binds to a

second nucleotide molecule of the kinase. The second nucleotide molecule may be mRNA, cDNA, sense DNA, anti-sense DNA, single-stranded and double-stranded DNA.

The invention includes a method of treating a disease in a mammal by using an inhibitor of a serine/threonine kinase, by using a natural or mimetic substrate for a serine/threonine kinase, or by using a first nucleotide molecule which binds to a second nucleotide molecule of the kinase. The method may include gene therapy, for example, the delivery of a gene or cDNA by any vector (viral or non-viral). The disease may be one selected from a group consisting of cancer, leukemia, solid tumors, chronic inflammatory disease, arthritis, osteoporosis and cardiovascular disease. The carrier for the inhibitor, substrate or molecule would be a pharmaceutically acceptable carrier, diluent or excipient. In the case of a nucleotide molecule, a carrier could be liposomes.

Diagnostics of ILK activity are part of this invention. Diagnostics include nucleotide molecules of ILK, ILK or its inhibitors. DNA-based reagents derived from the nucleotide sequence of ILK and antibodies against ILK screen biopsy-derived samples of amplified ILK DNA, or increased expression of ILK mRNA or protein.

Assays which screen drugs which specifically inhibit ILK activity are included within this invention. These assays may be based on the DNA, mRNA or amino acid sequences of IL-K.

The invention includes a pharmaceutical comprising an inhibitor of ILK activity together with a carrier, for modulating cellular activity.

Figures

FIG. 1 Yeast two-hybrid cloning, characterization, and expression of ILK. **a**, The full length ILK cDNA. **b**, Homology with protein kinase subdomains I to XI. **c**, Amino acid residues comprising ankyrin repeats. **d**, BIT-9 used to probe RNA from human tissues. **e**, Analysis of whole cell lysates of mouse, rat and human cell lines.

FIG. 2 *In vitro* and immune-complex kinase assays. **a**, *In vitro* kinase reactions. **b**, Immune complexes. **c**, ^{32}P-labelled products isolated and analyzed for phosphoamino acid content.

FIG. 3 Antibodies to GST-ILK^{132} recognize p59^{ILK} in integrin co-immunoprecipitations. **a**, Unfractionated polyclonal anti-ILK sera specifically recognize a ^{35}S-methionine, metabolically-labelled cellular protein. **b**, Affinity-purified antibody was adsorbed with GST-ILK agarose-GST. **c**, Polyclonal anti-integrin antibodies used to precipitate surface-biotinylated integrins from PC3 cells,. **d**, Anti-β_1 monoclonal antibodies were used in co-precipitation analyses of lysates of PC3.

FIG. 4 Modulation of ILK kinase activity by ECM components. **a**, ILK phosphorylation of MBP was assayed. **b**, Expression levels of p59^{ILK}. **c**, Representative p59^{ILK} overexpressing clone ILK13-A4a on the ECM substrates. **d**, Adhesion of the ILK overexpressing clones to LN, FN and

VN was quantified. **e**, ILK13, $p59^{ILK}$ overexpressing clones were assayed for colony growth.

FIG. 5 Expression of ILK in human breast carcinomas. **a**, Normal region of breast tissue. **b**, Ductal carcinoma *in situ*. **c,d**, Invasive carcinoma.

Detailed Description of Preferred Embodiments

By this invention, we have shown the physical linkage between integrin and ILK. More importantly, we have shown that dysregulated expression of ILK protein modulates the function of integrins, thus providing a biological link between ILK and integrin. Dysregulated expression of ILK modulates cell growth, cell adhesion, cell migration and cell invasion. Hence, products that inhibit the activity of dysregulated expression of ILK have a therapeutic effect in the treatment of cancer, leukemia, solid tumors, chronic inflammatory disease, arthritis and osteoporosis, among other indications.

The ILK protein is encoded by a 1.8 kilobase pair messenger RNA (1.8 kb mRNA). The sequence of this mRNA was used to deduce the primary amino acid sequence of the protein, which has a predicted molecular weight of 50 kiloDaltons (kDa). The recombinant protein migrates on analytical polyacrylamide electrophoresis gels with an apparent molecular weight of 59 kDa, in rough agreement with the predicted size. The deduced structure of the ILK protein (hereinafter p59ILK) revealed two functional domains, identified by comparison of the ILK sequence against those found in current protein databases. These are the catalytic domain, responsible for phosphotransferase activity (kinase domain), and a non-

overlapping domain in the amino terminus, comprised of four contiguous ankyrin-like.

The function of ankyrin repeats in ILK is to mediate protein-protein interactions. The ILK ankyrin repeat domain is not required for the binding of p59ILK to integrin, and it presumably mediates the interaction of p59ILK with another cellular protein(s). Thus, p59ILK bridges integrin(s) in the plasma membrane, with intracellular proteins active in regulating the cell's response to ECM signals. These proteins are likely to be located in the cytoplasm, or as part of the cell's structural framework (cytoskeleton), but are as yet unidentified.

The novelty of ILK lies in key structural and functional features of the enzyme. Structurally, it represents an unusual molecular architecture, in that a protein kinase and an ankyrin repeat domain are contained within the same protein. The kinase domain is very conserved (i.e. similar) to other kinase sequences in existing databases, and can be divided into typical subdomains (I through XI), based on this conserved structure. However one amino acid in particular is not present in ILK, which is present in a specific context, in subdomain VIb of all other protein kinase domains. Despite this unique structural feature, ILK clearly acts as a protein kinase, and thus could represent a prototype member of a new subfamily of protein kinase molecules.

The commercial potential of ILK is directly linked to its regulation of integrin extracellular activity (ECM interactions) from inside the cell, via its direct

interaction with the integrin subunit (known in the integrin field as "inside-out" signalling). Interfering with ILK activity allows the specific targeting of integrin function, while leaving other essential signalling pathways intact. Moreover, increasing the levels of cellular ILK activity short circuits the normal requirement for adhesion to ECM (i.e. integrin function) in regulating cell growth. Thus, inhibiting ILK activity would inhibit anchorage-independent (i.e. cancerous) cell growth.

Thus, from a therapeutics point of view, inhibiting ILK activity has a therapeutic effect on a number of proliferative disorders, including inflammation and cancer. Inhibition is achieved in a number of ways: (1) with screens aimed at DNA, RNA of ILK or ILK structural components e.g. antisense ILK (i.e. synthetic DNA oligonucleotide comprising the complementary nucleotide sequence of the ILK coding region, designed to specifically target the ILK mRNA complement), (2) pseudo-substrate inhibitors, for example, a peptide which mimics a substrate for ILK, or (3) by assaying inhibition of ILK activity in an ILK-based functional assay e.g. in vitro or in vivo ILK kinase activity.

Knowledge of the 3-dimensional structure of ILK, derived from crystallization of purified recombinant ILK protein, leads to the rational design of small drugs which specifically inhibit ILK activity. These drugs may be directed at either the kinase or ankyrin repeat domains.

From a diagnostics perspective, DNA-based reagents derived from the sequence of ILK, e.g. PCR primers, oligonucleotide or cDNA probes, as well as antibodies against p59ILK, are used to screen biopsy-derived tumours or inflammatory samples e.g. arthritic synovium, for amplified ILK DNA, or increased expression of ILK mRNA or protein. DNA-based reagents are designed for evaluation of chromosomal loci implicated in certain diseases e.g. for use in loss-of-heterozygosity (LOH) studies, or design of primers based on ILK coding sequence.

Having mapped the ILK chromosomal locus to region 11p15, it was determined that a subset of breast carcinomas displays LOH for markers in chromosomal region 11p15.5. This region has also been implicated in an inherited form of cardiac arrythmia, the long QT syndrome. A high level of expression of ILK mRNA indicates an integrin-independent function for ILK in cardiac tissue.

Example 1 Isolating cDNA of ILK and ILK

A partial cDNA, BIT-9, was isolated in a two-hybrid screen[4] using a bait plasmid expressing the cytoplasmic domain of the β_1 integrin subunit. The BIT-9 insert was used to isolate clones from a human placental cDNA library. A 1.8 kb clone, Plac5, was found to contain a high degree of similarity to cDNAs encoding protein kinases (Figure 1 a-c), and recognized a widely expressed transcript of 1.8 kb in Northern blots (Figure 1 d). Deduced amino acid residues 186-451 from Plac5 comprise a domain which is highly homologous with the catalytic domains of a large number of protein tyrosine and

serine/threonine kinases (Figure 1b). Residues 33-164 comprise four repeats of a motif originally identified in erythrocyte ankyrin[5] (Figure 1 c), likely defining a domain involved in mediating additional protein-protein interactions.[6, 7] Affinity-purified anti-ILK antibodies (see methods described in Example 3) were used in Western blot analyses of mammalian cell extracts, and detected a conserved protein of apparent Mr of 59 kDa ($p59^{ILK}$, Figure 1 e).

Figure 1 shows yeast two-hybrid cloning, characterization, and expression of ILK. **a**, The full length ILK cDNA, Plac5, was isolated from a human placental library using the BIT-9 insert. Plac5 contains a 1509 bp open reading frame, with a presumptive initiator Met^{17} at nt 157, and an AAUAAA signal 11 bp upstream of the polyadenylation site. *In vitro* transcription and translation of Plac5 in rabbit reticulocyte lysates yielded a protein of apparent Mr of 59 kDa (not shown). **b**, A search[18] of the PIR protein database indicated homology with protein kinase subdomains I to XI, as identified by Hanks et al.[19] We note sequence variations in the ILK subdomains I, VIb, and VII, relative to catalytic domains of known protein kinases. Subdomain I (residues 199-213), does not have the typical GXGXXG motif, although this region in ILK is Gly-rich. In subdomain VIb, Asp^{328} of ILK may compensate for the lack of the otherwise conserved Asp^{319}. In subdomain VII, the DFG triplet is absent in ILK. The integrin binding site maps to amino acid residues 293-451

(BIT-9). The ILK kinase domain is most highly related to the CTR1 kinase of *Arabidopsis thaliana* (30% identity, $P < 10^{-13}$). The CTR1, B-raf, Yes and Csk kinase domains are aligned with Plac5. **c**, Amino acid residues 33-164 comprise four contiguous ankyrin repeats, as defined by Lux et. al.[5] **d**, BIT-9 was used to probe a blot of poly A+ selected RNA (MTN I, Clontech) from various human tissues. **e**, Whole cell lysates of mouse, rat and human cell lines (10 µg/lane) were analyzed by Western blotting with the affinity-purified 92-2 antibody (see description of methods in Example 3). The ILK sequence data are available from GenBank under accession number U40282.

In order to construct integrin 'bait' plasmids[20], sequences encoding amino acid residues 738-798 of the β_1, and residues 1022-1049 of the α_5 integrin subunits were amplified from full-length cDNAs.[21] The primers used were (a) 5' amplification $^{5'}$ - GGCCGAATTCGCTGGAATTGTTCTTATTGGC -3' and (b) 3' amplification$^{5'}$ - GGCCGGATCCTCATTTTCCCTCATACTTCGG -$^{3'}$. PCR products were directionally cloned into pEG202, creating the LexA fusion bait plasmids, pEG202β_1INT and pEG202α_5INT. pEG202β_1INT and pEG202α_5INT repressed β-gal expression from the pJK101 reporter by 50-60% and 70-75%, respectively, in host strain EGY48 (MATα , his3, trp1, ura3-52, LEU2::pLEU2-LexAop6, constructed by Erica Golemis, Massachussetts General Hospital), confirming nuclear expression of the LexA fusions. Co-transformation of baits with the pSH18-34 reporter verified they were transcriptionally inert (not shown). A galactose-

inducible HeLa cDNA interactor library was present on the TRP+ vector, pJG4-5 (constructed by Jeno Gyuris, MGH). For the β_1 interaction trap, EGY48 was transformed sequentially with pEG202β_1INT, pSH18-34 and pJG4-5, using the lithium acetate protocol[22] (transformation efficiency = 5-6 x $10^4/\mu g$). 2x10^6 primary transformants were screened, of which forty-nine interacting clones were confirmed. The most frequent isolate (31/49) was a 700 bp insert, BIT-9. Retransformation of EGY48 with the BIT-9, pSH18-34, and pEG202β_1INT plasmids resulted in strong β-galactosidase expression, confirming the interaction. An identical screen, using pEG202α_5INT as bait, resulted in the isolation of 16 positives, none of which were represented in the set of 49 β_1 interactors. Trapped inserts were used to screen WM35 human melanoma λgt10, and human placental λgt11 cDNA libraries, using standard procedures.[23] cDNA sequencing of multiple clones from each library was done using the dideoxy chain termination method (Sequenase 2.0, U.S. Biochemical). For data analysis we used the Genetics Computer Group software package (version 7.0), and database searches were accomplished via the BLAST[18] server at the National Center for Biotechnology Information.

Example 2 Analysis of ILK In Vitro

For analysis of kinase activity *in vitro*, a bacterially-expressed fusion protein, GST-ILK^{132}, was SDS-PAGE band purified, and incubated with [γ-^{32}P]ATP in the presence or absence of the exogenous substrate myelin

basic protein (Figure 2). GST-ILK^{132} autophosphorylated and labelled MBP efficiently in these assays (Figure 2 a). Anti-GST-ILK^{132} (antibody 91-3) immunoprecipitates of PC3 cell lysates were incubated with [γ-^{32}P]ATP, similar to experiments performed with purified recombinant GST-ILK^{132}. ILK immune complexes labelled a protein of apparent Mr of 59kDa (Figure 2 b), corresponding to p59^{ILK}, as well as cellular proteins of apparent Mr 32 kDa and 70 kDa, which may be endogenous ILK substrates (Figure 2 b). We also see cellular phosphoproteins (serine/threonine) of approximately 32 kDa and 70 kDa, in β_1 integrin-specific immune complex kinase assays (not shown).

In ILK immune complex kinase assays a synthetic peptide representing the β_3 cytoplasmic domain was phosphorylated, while a similar peptide representing the β_3 cytoplasmic domain was not detectably labelled by p59^{ILK}. The β_1 peptide selectively inhibited autophosphorylation of ILK in these reactions (Fig. 2b), further indicating a differential interaction of the peptides with ILK. The results demonstrating phosphorylation of synthetic β peptides by endogenous ILK are identical to those seen with recombinant GST-ILK^{132} (not shown), and indicate the potential substrate preference of ILK for the β_1 cytoplasmic tail. This does not, however, necessarily rule out an interaction between ILK and the β_3 integrin cytoplasmic domain. Phosphoamino acid analyses, of labelled p59^{ILK} and MBP from the immune

complex kinase assays detected only phosphoserine in both substrates (Fig. 2 c), as was the case for phosphorylation of these substrates by GST-ILK^{132} (not shown). The β_1 peptide was labelled on serine and threonine residues, with approximately equal stoichiometry (Figure 2). As a control, anti-FAK[8, 9] immune complexes from the same lysates were analyzed for phosphorylation of MBP, and phosphotyrosine was readily detected (not shown).

Figure 2 shows *in vitro* and immune-complex kinase assays. **a**, *In vitro* kinase reactions containing 2 µg of gel-purified GST-ILK^{132} , with and without 5 µg of myelin basic protein (MBP, Upstate Biotechnologies, Inc.), were analyzed by 10% SDS-PAGE. **b**, Immune complexes were generated from PC3 whole cell lysates, using affinity-purified 91-3 antibody. Complexes were assayed for kinase activity, with and without addition of 5 µg/reaction of synthetic peptides, representing β_1 or β_3 integrin cytoplasmic domains,[24] or MBP (not shown). Products were analyzed by 15% SDS-PAGE (kDa markers at left), and migration of peptides confirmed by Coomassie Blue staining. **c**, ^{32}P-labelled products from the anti-ILK immune complex kinase reactions shown in b, were isolated and analyzed for phosphoamino acid content. Anti-FAK[8, 9] immune complex kinase assays demonstrated phosphotyrosine on MBP (not shown).

Protein kinase assays were performed in 50 µl kinase reaction buffer (50 mM HEPES pH 7.0, 10 mM $MnCl_2$, 10 mM

$MgCl_2$, 2 mM NaF, 1 mM Na_3VO_4), containing 10 µCi [γ-^{32}P]ATP. Reactions were incubated at 30°C for 20 min, and stopped by the addition of SDS-PAGE sample buffer. For assay of recombinant ILK activity, GST-ILK^{132} was adsorbed from bacterial lysates onto glutathione-agarose beads, or GST-ILK^{132} was band-purified from 10% SDS-PAGE gels. For immune complex kinase assays, affinity-purified 91-3 anti-ILK antibody (Fig. 3, Methods) was used to generate immunoprecipitates from NP-40 lysates (150 mM NaCl, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 50 mM HEPES pH 7.5, 1 µg/ml each leupeptin and aprotinin, 50 µg/ml phenyl-methylsulfonyl flouride) of PC3 cells. Kinase reaction products were resolved on 10-15% SDS-PAGE gels, transferred to PVDF, and phosphoamino acid analysis performed according to a published protocol.[25]

Example 3 Association of ILK and b integrin In Mammalian Cells

Immunofluorescence experiments indicated that ILK and β integrin co-localize in focal plaques (not shown). In order to test further for this association in intact mammalian cells, we performed co-immunoprecipitation assays in lysates of PC3 cells, in which integrin expression has been well-characterized.[10] PC3 cell lysates were immunoprecipitated with specific anti-integrin antibodies,[13] and immune complexes analyzed by Western blotting with the anti-ILK antibody, 92-2. The specificities of the anti-ILK antibodies were tested by immunoprecipitation and Western blotting (Figure 3 a, b).

We detected $p59^{ILK}$ in immune complexes obtained with anti-fibronectin receptor (FNR, α_5/α_3 β integrin), and anti-vitronectin receptor (VNR, $\alpha_v\beta_3/\beta_5$ integrin) antibodies, but not in those obtained with non-immune serum (Figure 3 c). Three anti-β_1 monoclonal antibodies also co-precipitated $p59^{ILK}$ from PC3 lysates, confirming the β integrin specificity of $p59^{ILK}$ interaction (Figure 3 d). The detection of $p59^{ILK}$ in anti-VNR immune complexes suggests that ILK may also interact with the β_3 and/or β_5 integrin subunit(s).

Figure 3 shows that antibodies to GST-ILK^{132} recognize $p59^{ILK}$ in integrin co-immunoprecipitations. **a**, Unfractionated polyclonal anti-ILK sera 91-3 (shown) and 92-2 specifically recognize a ^{35}S-methionine, metabolically-labelled cellular protein, of apparent Mr of 59 kDa. A fluorograph is shown (En^{3}Hance, NEN). **b**, Affinity-purified 92-2 antibody was adsorbed with 165 µg of agarose-coupled GST-ILK^{132}, or agarose-GST, which preparations were used in parallel Western blots containing 10 µg/lane of whole cell lysates of PC3 cells, Jurkat T-lymphoblasts, or the 60 kDa GST-ILK^{132}. **c**, Polyclonal anti-integrin antibodies, specific for the fibronectin and vitronectin receptors, were used to precipitate surface-biotinylated integrins from PC3 cells, and immune complexes were then analyzed for the presence of $p59^{ILK}$, by Western blotting with affinity-purified, biotin-labelled 92-2 antibody. This result is

representative of six independent experiments. d, Anti-β_1 monoclonal antibodies were used in co-precipitation analyses of NP-40 lysates of PC3: lane 1, $A_{II}B_2$; lane 2, anti-CD29; lane 3, 3S3. Western blotting of anti-β_1 immune complexes with affinity-purified, biotinylated 92-2 antibody (left). This blot was stripped and reprobed with the same concentration of biotinylated 92-2, adsorbed against an excess of GST-ILK^{132} beads (right). We observe co-precipitation of p59^{ILK} using a panel of 11 anti-b_1 monoclonals, but not with an anti-CD44 monoclonal antibody (not shown). The migration of p59^{ILK} was confirmed in parallel lanes containing PC3 whole cell NP-40 lysates. Markers at left, in kDa.

Amino acid residues 132-451 of ILK were expressed as a GST fusion protein, in *E. coli*. Recombinant GST-ILK^{132} protein was purified and used to inject two rabbits. The resulting antisera, 91-3 and 92-2 (raised by Research Genetics, Inc.), were affinity-purified over a column of CNBr-Sepharose coupled GST-ILK^{132}. PC3 cells were metabolically labelled with 100 µCi/ml [^{35}S]methionine/ [^{35}S]cysteine ([^{35}S] ProMix, 1000 Ci/mmol, Amersham), for 18 hours in cysteine/methionine-free MEM. For co-immunoprecipitation experiments PC3 cells were surface-labelled with sulfo-NHS-biotin[26] (Pierce Chemicals), prior to lysis in NP-40 buffer. Polyclonal anti-fibronectin receptor (anti-FNR, Telios A108), and anti-vitronectin receptor (anti-VNR, Telios A109) antibodies were purchased from Gibco/BRL. 1-2 mg of NP-40 lysate was

incubated at 4°C, with 2-3 µl/ml anti-FNR or anti-VNR antiserum, or 2 µg/ml of the anti-β_1 monoclonal antibodies $A_{II}B_2$ (C. Damsky, UC, San Francisco), anti-CD29 (Upstate Biotechnology, Inc.), and 3S3 (J. Wilkins, U Manitoba). Lysates were pre-cleared and immune complexes collected with, Protein A-Sepharose. For Western blotting, RIPA[27] lysates or immune complexes were subjected to 7.5% or 10% SDS-PAGE, and proteins then electrophoretically transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore). Membranes were blocked in 5% non-fat milk/Tris-buffered saline Tween-20, and incubated with 0.5 µg/ml affinity purified antibodies. Horseradish peroxidase-coupled goat anti-rabbit IgG was used in secondary incubations, followed by detection of reactive bands by enhanced chemiluminescence (ECL, Amersham). For blotting without use of secondary antibody (Fig. 3), affinity-purified 92-2 antibody was labelled with Biotin Hydrazide (Immunopure, Pierce Chemicals), according to the manufacturer's protocol, with visualization by peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories) and ECL. For re-probing, membranes were stripped according to manufacturer's instructions.

Example 4 Overexpression of ILK Provides Growth Advantage

We next tested for fibronectin-dependent regulation of ILK kinase activity. Plating of rat intestinal epithelial cells, IEC-18,[11] on fibronectin reduced ILK phosphorylation of MBP in immune complex kinase assays, relative to cells plated on plastic, or kept in

suspension (Figure 4 a). This fibronectin-dependent reduction of ILK activity was abrogated in IEC-18 cells expressing an activated H-*ras* allele,[11] indicating that *ras* transformation disrupts ECM regulation of ILK activity in these cells. An expression vector containing the full-length ILK cDNA, pCMV-ILK, was stably transfected into IEC-18 cells. Twelve stable clones each, of pCMV-ILK and vector control transfectants, were selected and characterized for $p59^{ILK}$ expression levels. Two representative overexpressing subclones, ILK13-A1a3 and -A4a are illustrated (Figure 4b). Overexpression of $p59^{ILK}$ disrupted the epithelial morphology of IEC-18 cells. ILK13 clones were more refractile, and grew on LN, FN and VN with a stellate morphology, in marked contrast to the typical, 'cobble-stone' morphology of the parental and ILK14 cells (Figure 4 c). We plated the ILK13-A1a3 and -A4a subclones, the control transfectants, ILK14-A2C3 and -A2C6, and IEC-18 cells, on varying concentrations of the integrin substrates, laminin (LN), fibronectin (FN) and vitronectin (VN). Adhesion of the ILK14 and IEC-18 cells was equivalent, whereas that of the overexpressing subclones was significantly reduced, on all these substrates (Figure 4 d). Immunoprecipitation analysis indicated that cell surface integrin expression was unaffected (not shown). The effect of $p59^{ILK}$ overexpression on anchorage-independent growth was examined by assaying the colony forming ability of ILK transfectants in soft agarose. In marked contrast to IEC-18 and transfectant controls, four independent $p59^{ILK}$ overexpressing subclones, ILK13- A4a, A1a3, A4d3 and

A4C12, formed colonies in these assays (Figure 4 e). The proliferative rates of all of these clones on tissue culture plastic were equivalent to control rates.

Figure 4 shows the modulation of ILK kinase activity by ECM components. **a**, ILK phosphorylation of MBP was assayed in ILK immune complexes, from lysates of IEC-18 intestinal epithelial cells which were harvested from tissue culture plastic and either kept in suspension, or replated on fibronectin, for 1 hour. A H-*ras*-transformed variant of IEC-18,[11] Ras37 (transfected with Ras^{val12} in pRC/CMV vector), was assayed in parallel. The band shown is MBP. **b**, Expression levels of $p59^{ILK}$ in two representative clones of IEC-18 cells, transfected with an ILK expression construct (ILK13), two vector control clones (ILK14), and the parental IEC-18 cells are presented. The indicated amounts (µg/lane) of whole cell RIPA lysates were run out on 10% SDS-PAGE gels, and $p59^{ILK}$ expression analyzed by Western blotting with affinity-purified 92-2 antibody. **c**, Representative $p59^{ILK}$ overexpressing clone ILK13-A4a, vector control clone ILK14-A2C3, and parental IEC-18 cells were plated on the ECM substrates LN, FN and VN for 1 hour, then fixed, stained with toluidine blue and photographed (40x mag). **d**, Adhesion of the ILK overexpressing clones to LN, FN and VN was quantified. Key: IEC-18 (black), ILK14-A2C6 (white), ILK13-A1a3 (dark grey), ILK13-A4a (light grey). Results are presented for 10 µg/ml substrate, and are expressed as % adhesion (+/- s. d.) relative to IEC-18, for each substrate. The serial concentrations of ECM showed similar reductions in adhesion of the ILK13

subclones, and ILK14-A2C3 adhesion was identical to that of ILK14-A2C6, on all three substrates. Immunoprecipitation of surface-biotinylated IEC-18, ILK13, and ILK14 subclones, with the anti-FNR and anti-VNR sera, confirmed there was no change in expression of $\alpha_5/\alpha_3\beta_1$ and $\alpha_v\beta_3/\beta_5$ integrin subunits in the $p59^{ILK}$ overexpressors (data not shown). Data are representative of two independent experiments. e, Four ILK13, $p59^{ILK}$ overexpressing clones were plated in soft agarose, and assayed for colony growth after three (experiment 1) and two (experiment 2) weeks. Parent and vector control transfectants were also assayed, and the ras^{val12} transformed clone, Ras-37, was used as a positive control. Bars represent the mean of duplicate determinations. Maximum colonies in IEC-18 and ILK14 cells was 1/field.

The rat intestinal epithelial cell line IEC-18, and a variant of this line transfected with an activated H-ras^{val12} allele, expressed from pRC/CMV, were grown on tissue culture plastic in 5% serum-containing medium, washed three times in minimum essential medium (MEM), and harvested with 5 mM EDTA. These were resuspended in 2.5 mg/ml BSA in MEM, and either kept in suspension, or plated on 10 µg/ml fibronectin-coated plates, for 1 hour at 37°C. NP-40 lysates (300 µg) of these cells were immunoprecipitated with affinity-purified 91-3, and immune complex kinase assays (MBP substrate) performed, as described above. IEC-18 were transfected with the expression vector pRC/CMV, containing Plac5 in the forward orientation relative to the CMV promotor. Stable

clones were selected in G418, and subcloned through two rounds of limiting dilution. In all, twelve each of ILK and vector control transfectant subclones were isolated. Protein concentrations were determined using the Bradford reagent (Bio-Rad). Two $p59^{ILK}$ overexpressors, ILK13-A1a3 and ILK13-A4a, and two vector transfectant controls, ILK14-A2C3 and -A2C6, were analyzed for effects of ILK overexpression on cell adhesion to ECM substrates. Adhesion was quantified according to published methods.[28] For colony formation assays 3×10^5 cells were plated in 35mm wells, in 0.3% agarose, as described previously.[29] Ras-37 were plated at 2×10^3/well. Colonies were counted and scored per field (d = 1 cm) in duplicate wells, and defined as a minimum aggregate of 50 cells.

These results demonstrate that $p59^{ILK}$ overexpression in the IEC epithelial cells provides a growth advantage, in the absence of proliferative signals normally provided by adhesion.

The transduction of extracellular matrix signals through integrins influences intracellular ('outside-in') and extracellular ('inside-out') functions, both of which appear to require interaction of integrin cytoplasmic domains with cellular proteins.[12, 13] The association of ILK with β_1 integrin subunits, and specific regulation of its kinase activity by adhesion to fibronectin, suggests that $p59^{ILK}$ is a mediator of integrin signalling. Thus the ankyrin repeat motif likely represents a protein interaction module specifying interactions of ILK with

downstream, cytoplasmic or cytoskeletal proteins. Reduced ECM adhesion by the $p59^{ILK}$ overexpressing cells is consistent with our observation of adhesion-dependent inhibition of ILK activity, and suggests that $p59^{ILK}$ plays a role in inside-out integrin signalling. Furthermore the $p59^{ILK}$-induced, anchorage-independent growth of epithelial cells indicates a role for ILK in mediating intracellular signal transduction by integrins.[14-16]

Example 5 The Effect of Anti-ILK On Cell Migration

The role of ILK in cell motility has important implications for normal physiological processes such as inflammation and wound healing, as well as pathological conditions involving tumour invasiveness and metastatic tumour spread, or osteoporosis (bone is essentially an extracellular matrix secreted by osteoblast, or bone-forming cells, and this deposition can be modulated by integrin expression levels and function). Cell motility is a dynamic process, which is dependent on integrin-ECM interactions. The "on-off" switch function of protein kinases provides an ideal mechanism for the dynamic regulation of integrin affinity states for ECM substrates. Thus we are currently assaying the effect on cell migration of microinjecting highly specific anti-ILK antibodies (thereby inhibiting ILK function) into the cell's cytoplasm. Initially these effects will be assayed in endothelial cells plated on solid substrata, but will be extended to include studies on cell migration through three-dimensional gels composed of ECM proteins.

Example 6 Anti-Sense Oligonucleotides to Inhibit ILK Activity

The sequence of ILK cDNA provides information for the design and generation of synthetic oligonucleotides for "anti-sense" inhibition of ILK activity. This term derives from the strategy of employing a reverse complement of the coding, or sense strand of a specific messenger RNA, known as an anti-sense oligonucleotide (AO). By binding to its complementary mRNA, the AO inhibits translation of that mRNA into protein, thereby preventing normal protein accumulation in the cell. It is not possible to predict which region of an mRNA will provide the most efficient translational inhibition, although we will test ILK AO derived from the ILK mRNA sequence closest to the presumptive translational start site, as defined in Fig.1, as this provides the most successful reagents for this.

Regardless of the actual chemistry used to construct the AO, or modifications to an anti-ILK AO to improve its efficiency, the cDNA sequence of ILK provides the information for derivation of a specific AO. The cDNA sequence of ILK is used to design oligonucleotide reagents, known as degenerate primers (due to the degeneracy of the genetic code), for use in polymerase chain reaction (PCR)-based screens for cDNAs structurally related to ILK. Similarly, the ILK cDNA is used to screen for related genes in a more conventional screen of genomic or cDNA libraries, by employing less stringent (i.e. milder) hybridization conditions during screening. In this way, distinct cDNA or DNA sequences significantly related to ILK (> 50% nucleotide identity) can be

isolated, and a family of ILK-related kinases identified in a non-random fashion.

Example 7 Mapping of ILK Chromosomal Locus to Assess Imprinted Copies of Gene

We conduct higher resolution mapping of the ILK chromosomal locus through fluorescent in situ hybridization (FISH) to metaphase (i.e. separated and identifiable) human chromosomes has placed the ILK gene on chromosome 11p15. FISH is known to those skilled in the art. Finer resolution uses known marker genes in this region. The 11p15 region is indicates that certain genes (e.g. insulin-like growth factor 2, IGF2) have been shown to be imprinted (i.e. preferentially expressed from either the maternally or paternally-derived chromosomes). This imprinting effectively provides a functional deletion or "knock-out" of one of the two inherited copies of a gene. Thus mutation of the non-imprinted allele (copy) has a more profound outcome, since no compensatory activity is available from the imprinted allele. Also, 11p15 has been identified as a region subject to loss-of-heterozygosity, or LOH, in a subset of breast tumour patients. LOH results in the loss of one allele, for example by gene deletion, and is a mechanism underlying the contribution of a number of tumour suppressor genes to the development of various cancers (e.g. BRCA1 in breast, DCC in colon carcinoma, and RB1 in retinoblastoma).

Thus ILK cDNA sequence is used to develop DNA reagents for the diagnosis and prognostic indications of a significant subset of breast cancers, and these

reagents contribute to the molecular classification of such tumours. As mentioned above, the gene(s) on 11p15 contributing to some inherited cases of long QT syndrome are identified, and the candidacy of ILK as a causative gene for this cardiac condition are evaluated by looking for alterations in ILK gene structure, in families where 11p15 associations have been made.

Example 8 Induction of *in vivo* Tumorigenesis by Overexpression of ILK

Overexpression of ILK down-regulates E-cadherin which is an important epithelial cell adhesion molecule mediating cell-cell interactions (Dedhar et al., unpublished observations). The loss of E-cadherin induced by overexpression of ILK in epithelial cells suggests that ILK may promote tumorigenicity *in vivo*. To test this, we injected cells expressing varying levels of ILK into athymic nude mice subcutaneously. Mice were inoculated subcutaneously with the cells expressing high (ILK13-A1a3 and A4a) or low (IEC-18 and ILK14-A2C3) levels of ILK (10^7 cells/mouse in PBS). The mice were monitored for tumor formation at the site of inoculation after three weeks. Tumors arose within three weeks in 50% to 100% of the mice injected with the ILK13 cells (10^7 cells/mouse) that overexpress ILK, whereas no tumors were detected in the mice that were injected with the same number of the IEC-18 or ILK14 cells expressing lower levels of ILK (Table I). Thus, overexpression of ILK in these epithelial cells promotes tumor formation in vivo.

TABLE I: Tumorigenicity of ILK Overexpressing IEC-18 Cells

Cell Line	Number of Mice with Tumors at 3 weeks
IEC-18	0/6
ILK14-A2C3	0/6
ILK13-A1a3	6/6
ILK13-A4a	3/6

Example 9 Increased expression of ILK in human breast carcinoma

The expression of Integrin Linked Kinase in human breast carcinomas was determined by immunohistochemical straining of paraffin embedded sections from human breast cancer biopsies. Affinity purified anti-ILK polyclonal antibody was used followed by conjugated secondary antibody. The positive staining observed was completely abolished by absorption of the antibody to ILK-coupled sepharose beads. The photomicrographs represent sections from two tumor samples. A total of 30 samples have been examined so far. In every case ILK expression levels are markedly elevated in tumor tissue compared to normal ducts and lobules. Figure 5A shows a normal region showing well formed ducts with a single layer of epithelial cells. ILK staining is most prominent in epithelial cells. The stroma appears negative. Figure 5B shows ductal carcinoma *in situ* (DCIS). Multiple cell layers are present with markedly elevated ILK staining in the tumor cells. Invasive carcinoma is depicted in

figures 5C and 5D. There is markedly elevated expression of ILK compared to the normal tissue shown in figure 5A.

The present invention has been described in terms of particular embodiments found or proposed by the present inventor to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. For example, due to codon redundancy, changes can be made in the underlying DNA sequence without affecting the protein sequence. Moreover, due to biological functional equivalency considerations, changes can be made in protein structure without affecting the biological action in kind or amount. All such modifications are intended to be included within the scope of the appended claims.

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We Claim:

1. An isolated and purified serine/threonine kinase which is an integrin-linked kinase.

2. The serine/threonine kinase of claim 1 for a use selected from a group consisting of modulating cell growth, modulating cell adhesion, modulating cell migration and modulating cell invasion.

3. An isolated nucleotide molecule of the kinase of claim 1.

4. The molecule of claim 3 which is selected from a group consisting of mRNA, cDNA, sense DNA, anti-sense DNA, single-stranded DNA and double stranded DNA.

5. An isolated nucleotide molecule encoding an impaired amino acid sequence of a serine/threonine kinase which is an integrin-linked kinase.

6. An inhibitor of the serine/threonine kinase of claim 1.

7. The inhibitor of claim 6, wherein the inhibitor is an antibody.

8. The inhibitor of claim 6, wherein the inhibitor is a a natural or mimetic substrate for the integrin-linked kinase.

9. The inhibitor of claim 6, wherein the inhibitor is a first nucleotide molecule which binds to a second nucleotide molecule of the kinase.

10. The inhibitor of claim 8, wherein the second nucleotide molecule is selected from a group

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consisting of mRNA, cDNA, sense DNA, anti-sense DNA, single-stranded DNA and double stranded DNA.

11. A method of treating a disease in a mammal by using an inhibitor of a serine/threonine kinase.

12. A method of treating a disease in a mammal by using a natural or mimetic substrate for a serine/threonine kinase.

13. A method of treating a disease in a mammal by using a first nucleotide molecule which binds to a second nucleotide molecule of the kinase.

14. The method of claim 13, wherein the second nucleotide molecule is selected from a group consisting of mRNA, cDNA, sense DNA, anti-sense DNA, single-stranded DNA and double stranded DNA.

15. The method of claim 10, claim 11, claim 12 or claim 13 wherein the method modulates cellular activity and wherein the cellular activity is selected from a group consisting of cell growth, cell adhesion, cell migration and cell invasion.

16. The method of claim 10, further comprising administering to the mammal a pharmaceutical comprising the inhibitor and a carrier.

17. The method of claim 10, claim 11, claim 12 or claim 13 wherein the disease is one selected from a group consisting of cancer, leukemia, solid tumors, chronic inflammatory disease, arthritis, osteoporosis and cardiovascular disease.

18. A pharmaceutical comprising an inhibitor of the serine/threonine kinase of claim 1 together with a

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carrier, for modulating cellular activity, wherein the cellular activity is selected from a group consisting of cell growth, cell adhesion, cell migration and cell invasion.

19. The pharmaceutical of claim 18, wherein the inhibitor is a natural or mimetic substrate for the serine/threonine kinase.

20. A pharmaceutical comprising a first nucleotide molecule which binds to a second nucleotide molecule of the serine/threonine kinase of claim 1 together with a carrier, for modulating cellular activity, wherein the cellular activity is selected from a group consisting of cell growth, cell adhesion, cell migration and cell invasion.

21. A diagnostic kit comprising the nucleotide molecule of claim 3 or claim 4.

22. A diagnostic kit comprising the serine/threonine kinase of claim 1.

23. A diagnostic kit comprising the inhibitor of claim 6.

24. An assay for screening inhibitors of a serine/threonine kinase of claim 1 comprising the kinase of claim 1.

25. An assay for screening inhibitors of a serine/threonine kinase comprising the nucleotide molecule of claim 3 or claim 4.

```
gaattcatctgtcgactgctaccacgggagttccccggagaaggatcctgcagcccgagt 60
cccgaggataaagcttggggttcatcctccttccctggatcactccacagtcctcaggct 120
tccccaatccaggggactcggcgccgggacgctgctATGGACGACATTTTCACTCAGTGC 180
                                      M  D  D  I  F  T  Q  C   8
CGGGAGGGCAACGCAGTCGCCGTTCGCCTGTGGCTGGACAACACGGAGAACGACCTCAAC 240
R  E  G  N  A  V  A  V  R  L  W  L  D  N  T  E  N  D  L  N   28
CAGGGGGACGATCATGGCTTCTCCCCCTTGCACTGGGCCTGCCGAGAGGGCCGCTCTGCT 300
Q  G  D  D  H  G  F  S  P  L  H  W  A  C  R  E  G  R  S  A   48
GTGGTTGAGATGTTGATCATGCGGGGGGCACGGATCAATGTAATGAACCGTGGGGATGAC 360
V  V  E  M  L  I  M  R  G  A  R  I  N  V  M  N  R  G  D  D   68
ACCCCCCTGCATCTGGCAGCCAGTCATGGACACCGTGATATTGTACAGAAGCTATTGCAG 420
T  P  L  H  L  A  A  S  H  G  H  R  D  I  V  Q  K  L  L  Q   88
TACAAGGCAGACATCAATGCAGTGAATGAACACGGGAATGTGCCCCTGCACTATGCCTGT 480
Y  K  A  D  I  N  A  V  N  E  H  G  N  V  P  L  H  Y  A  C   108
TTTTGGGGCCAAGATCAAGTGGCAGAGGACCTGGTGGCAAATGGGGCCCTTGTCAGCATC 540
F  W  G  Q  D  Q  V  A  E  D  L  V  A  N  G  A  L  V  S  I   128
TGTAACAAGTATGGAGAGATGCCTGTGGACAAAGCCAAGGCACCCCTGAGAGAGCTTCTC 600
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Fig. 1a (continued on page 2/23)

```
C  N  K  Y  G  E  M  P  V  D  K  A  A  K  A  P  L  R  E  L  L    148
CGAGAGCGGGCAGAGAAGATGGGCCAGAATCTCAACCGTATTCCATACAAGGACACATTC   660
R  E  R  A  E  K  M  G  Q  N  L  N  R  I  P  Y  K  D  T  F    168
TGGAAGGGACCACCCGGCACTCGGCCCCGAAATGGAACCCTGAACAAACACTCTGGCATT   720
W  K  G  T  T  R  T  R  P  R  N  G  T  L  N  K  H  S  G  I    188
GACTTCAAACAGCTTAACTTCCTGACGAAGCTCAACGAGAATCACTCTGGAGAGCTATGG   780
D  F  K  Q  L  N  F  L  T  K  L  N  E  N  H  S  G  E  L  W    208
AAGGGCCGCTGGCAGGGCAATGACATTGTCGTGAAGGTGCTGAAGGTTCGAGACTGGAGT   840
K  G  R  W  Q  G  N  D  I  V  V  K  V  L  K  V  R  D  W  S    228
ACAAGGAAGAGCAGGGACTTCAATGAAGAGTGTCCCCGGCTCAGGATTTTCTCGCATCCA   900
T  R  K  S  R  D  F  N  E  E  C  P  R  L  R  T  F  S  H  P    248
AATGTGCTCCCAGTGCTAGGTGCCTGCCAGTCTCCACCTGCTCCTCATCTCTACTCTCATC   960
N  V  L  P  V  L  G  A  C  Q  S  P  P  A  P  H  P  T  L  I    268
ACACACTGGATGCCGTATGGATCCCTCTACAATGTACTACATGAAGGCACCAATTTCGTC  1020
T  H  W  M  P  Y  G  G  S  L  Y  N  V  L  H  E  G  T  N  F  V    288
GTGGACCAGAGCCAGGCTGTGAAGTTTGCTTTGGACATGGCAAGGGGCATGGCCTTCCTA  1080
V  D  Q  S  Q  A  V  K  F  A  L  D  M  A  R  G  M  A  F  L    308
CACACACTAGAGCCCCTCATCCCACGACATGCACTCAATAGCCGTAGTGTAATGATTGAT  1140
H  T  L  E  P  L  I  P  R  H  A  L  N  S  R  S  V  M  I  D    328
```

Fig. 1a (continued on page 3/23)

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GAGGACATGACTGCCCGAATTAGCATGGCTGATGTCAAGTTCTCTTTCCAATGTCCTGGT 1200
E  D  M  T  A  R  I  S  M  A  D  V  K  F  S  F  Q  C  P  G    348
CGCATGTATGCACCTGCCTGGGTAGCCCCCGAAGCTCTGCAGAAGAAGCCTGAAGACACA 1260
R  M  Y  A  P  A  W  V  A  P  E  A  L  Q  K  K  P  E  D  T    368
AACAGACGCTCAGCAGACATGTGGAGTTTTGCAGTGCTTCTGTGGGAACTGGTGACACGG 1320
N  R  R  S  A  D  M  W  S  F  A  V  L  L  W  E  L  V  T  R    388
GAGGTACCCTTTGCTGACCTCTCCAATATGGAGATTGGAATGAAGGTGGCATTGGAAGGC 1380
E  V  P  F  A  D  L  S  N  M  E  I  G  M  K  V  A  L  E  G    408
CTTCGTACCATCCCACCAGGTATTTCCCCTCATGTGTGTAAGCTCATGAAGATCTGCATG 1440
L  R  T  I  P  P  G  I  S  P  H  V  C  K  L  M  K  I  C  M    428
AATGAAGACCCTGCAAAGCGACCCAAATTTGACATGATTGTGCCTATCCTTGAGAAGATG 1500
N  E  D  P  A  K  R  P  K  F  D  M  I  V  P  I  L  E  K  M    448
CAGGACAAGtaggactggaaggtccttgcctgaactccagaggtgtcgggacatggttgg 1560
Q  D  K  *                                                     451
gggaatgcacctccccaaagcagcaggcctctggttgcctcccccgcctccagtcatggt 1620
actaccccagcctggggtccatccccttcccccatccctaccactgtgcgcaagaggggc 1680
gggctcagagctttgtcacttgccacatggtgtctcccaacatgggagggatcagccccg 1740
cctgtcacaataaagtttattatgaaaaaaaaaaaaaaaaaaaa                 1786
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Fig. 1a

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                                    I                  II
Csk     .NMKELKLLQ TIGKGEFGDV MLGDYRGN.K VAVKCIKNDA .TAQ....AF
Yes     IPRESLRLEV KLGQGCFGEV WMGTWNGTTK VAIKTLKPGT MMPEAFLQ..
Ctrl    IPWCDLNIKE KIGAGSFGTV HRAEWHGS.D VAVKILMEQD FHAE.RVNEF
B-raf   IPDGQITVGQ RIGSGSFGTV YKGKWHG..D VAVKMLNVTA PTPQQ.LQAF
Ilk     IDFKQLNFLT KLNENHSGEL WKGRWQGN.D IVVKVLDKVR DWSTRKSRDF    235

          III               IV                                V
Csk     LAEASVMTQ LRHSNLVQLL GVIVEE.KGG LYIVTEYMAK GSLVDYLRSR
Yes     ..EAQIMKK LRHDKLVPLY AVVSEE...P IYIVTEFMTK GSLLDFLKEG
Ctrl    LREVAIMKR LRHPNIVLFM GAVTQPP..N LSIVTEYLSR GSLYRLLHKS
B-raf   KNEVGVLRK TRHVNILLFM GYSTKP...Q LAIVTQWCEG SSLYHHLHII
Ilk     NEECPRLRI FSHPNVLPVL GACQSPPAPH PTLITHWMPY GSLYNVLHE.    283

                                    VIa                VIb
Csk     GRSV.LGGDC LLKFSLDVCE AMEYLEGN.. .NFVHRDLAA RNVLVS.E
Yes     EGKF.LKLPQ LVDMAAQIAD GMAYIERM.. .NYIHRDLRA ANILVG.D
Ctrl    GAREQLDERR RLSMAYDVAK GMNYLH.NRN PPIVHRDLKS PNLLV.DK
B-raf   ETKFEMI..K LIDIARQTAQ GMDYLHAK.. .SIIHRDLKS NNIFLH.E
Ilk     GTNFVVDQSQ AVKFALDMAR GMAFLH.TLE PLIPRHALNS RSVMI.DE    329
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Fig. 1b (continued on page 5/23)

```
                VII                                                VIII
Csk     DNVAKVSDFG LTK.....EA SSTQDTGKLP VKWTAPEALR ...EKKFSTK
Yes     NLVCKIADFG LARLIED.NE YTARQGAKFP IKWTAPEAAL ...YGRFTIK
Ctrl    KYTVKVCDFG LSRLKAS.TF LSSKSAAGTP .EWMAPEVLR ...DEPSNEK
B-raf   DLTVKIGDFG LATVKSRWSG SHQFEQLSGS ILWMAPEVIR MQDKNPYSFQ
Ilk     DMTARIS... MADVKFSFQC PGRM.YA..P .AWVAPEALQ KKPEDTNRSS   372

                IX                                          X
Csk     SDVWSFGILL WEIYSFGRVP YPRIPLKD.V VPRVEKGY..  KMDAPDGCPP
Yes     SDVWSFGILL TELVTKGRVP YPGMVNRE.V LEQVERGY..  RMPCPQGCPE
Ctrl    SDVYSFGVIL WELAT.LQQP WGNL.NPAQV VAAVGFKCK.  RLEIPRNLNP
B-raf   SDVYAFGIVL YELMT.GQLP YSNINNRDQI IFMVGRGYLS PDLSKVRSNC
Ilk     ADMWSFAVLL WELVTR.EVP FADLSNMEIG MK.VALEGL.  R.TIPPGISP   418

                                XI
Csk     AVYEVMKN   CWHLDAAMRP SFLQLREQLE HIKTHEL
Yes     SLHELMKL   CWKKDPDERP TFEYIQSFLE .......
Ctrl    QVAAIIEG   CWTNEPWKRP SFATIMDLLR PL.....
B-raf   PKAMKRLMAECLKKKRDERP LFPQILASIE LLARSLP
Ilk     HVCKLMKI   CMNEDPAKRP KFDMIVPILE KMQDK..                451
```

Fig. 1b

```
ANKYRIN      -G-TPLH-AA--GH---V--LL--GA--N----
CONSENSUS              A           D

                33
ANK1    HGFSPLHWACREGRSAVVEMLIMRGARINVMNR
ANK2    GDDTPLHLAASHGHRDIVQKLLQYKADINAVNE
ANK3    HGNVPLHYACFWGQDQVAEDLVANGALVSICNK
ANK4    YGEMPVDKAKAPLRELLRERAEKMGQNLNRIPY
                                          164
```

Fig. 1c

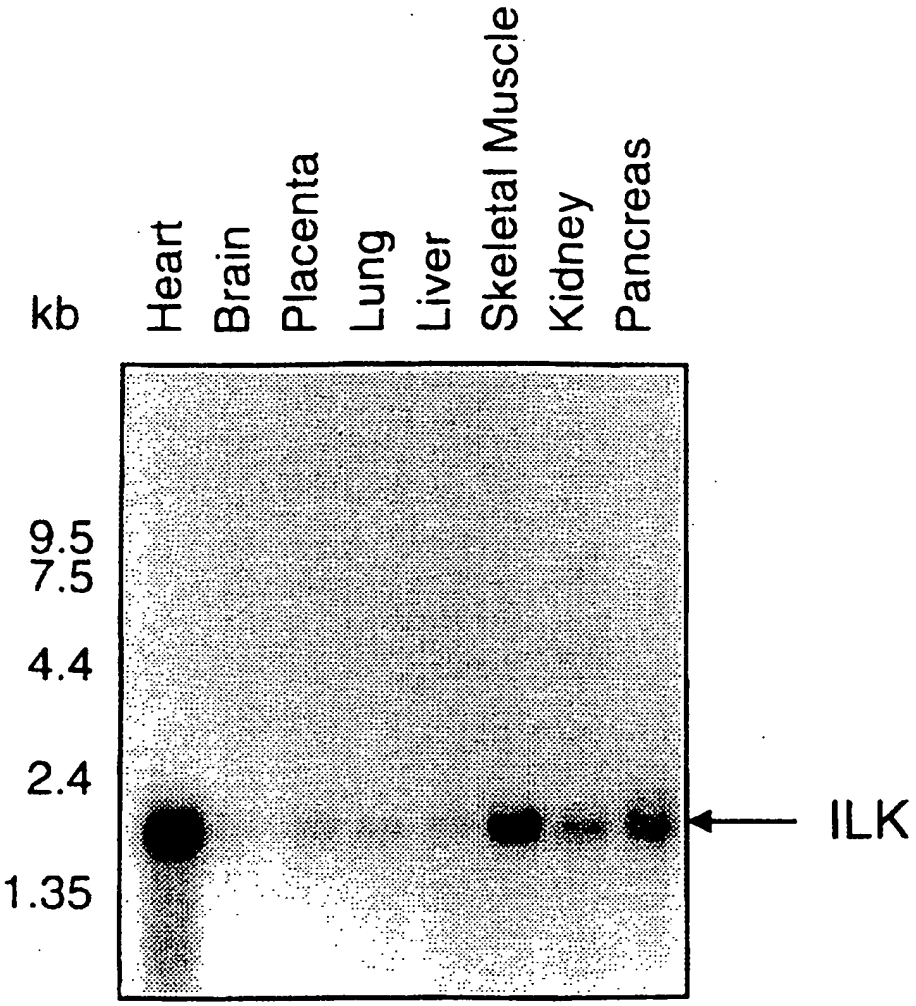
kb
Heart
Brain
Placenta
Lung
Liver
Skeletal Muscle
Kidney
Pancreas
9.5
7.5
4.4
2.4
1.35
ILK

Fig. 1d

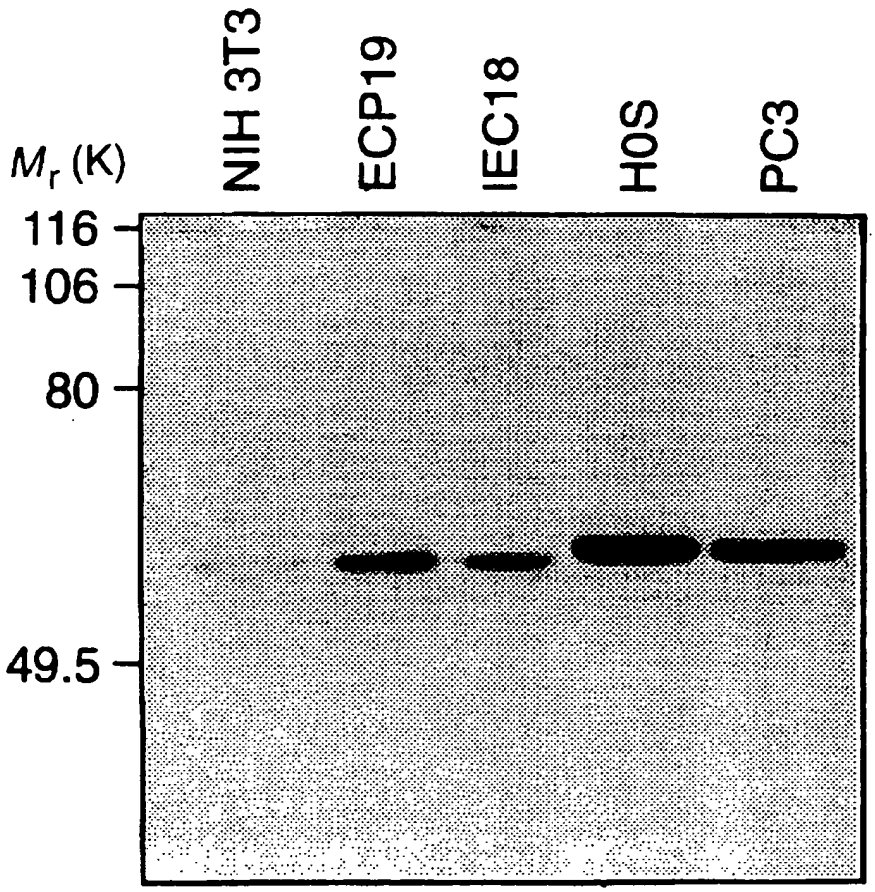

Fig. 1e

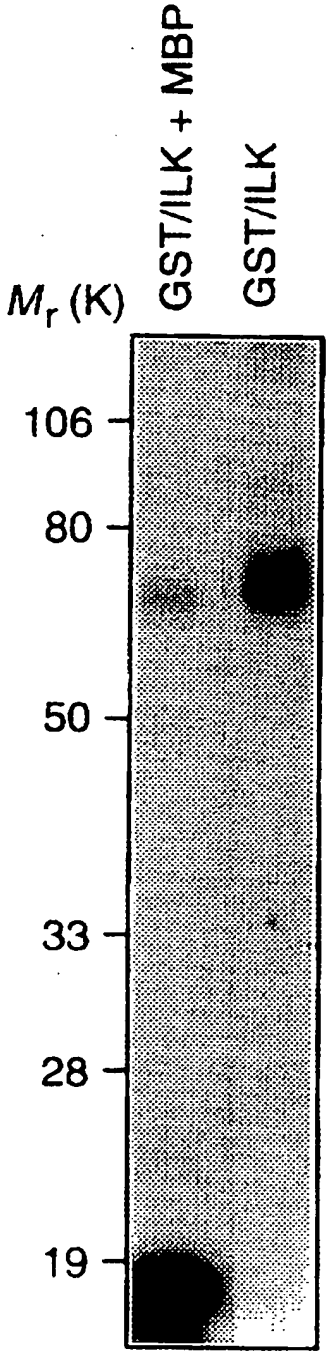

Fig. 2a

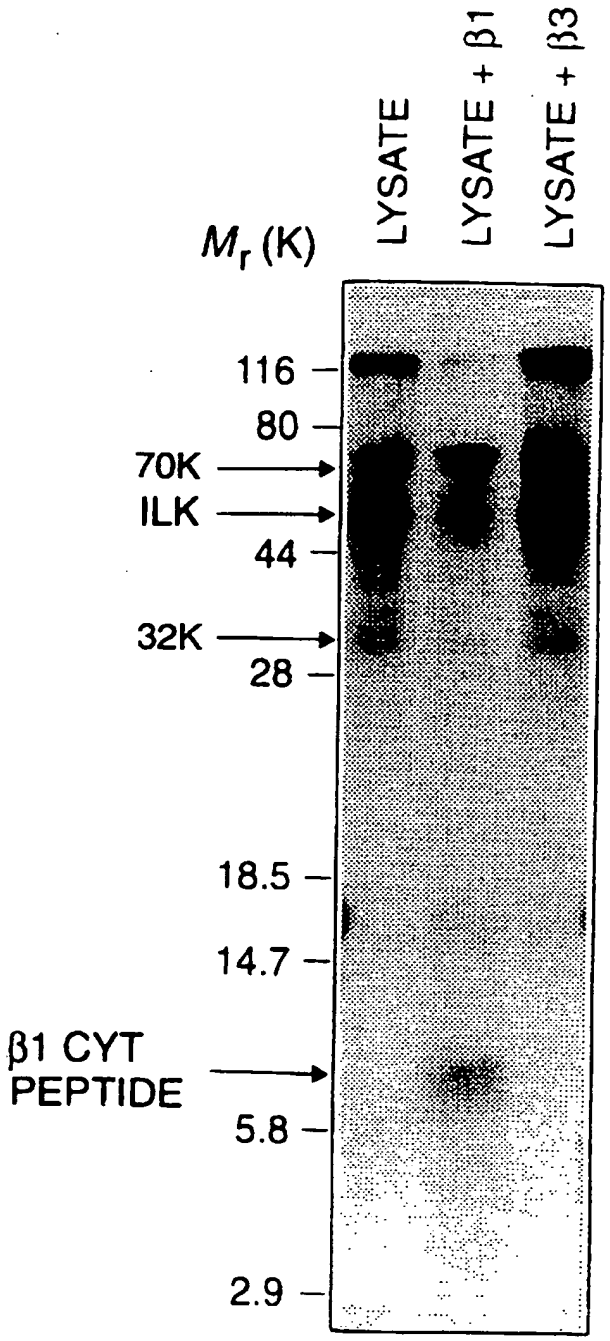
LYSATE
LYSATE + β1
LYSATE + β3
M_r (K)
116
80
70K
ILK
44
32K
28
18.5
14.7
β1 CYT PEPTIDE
5.8
2.9

Fig. 2b

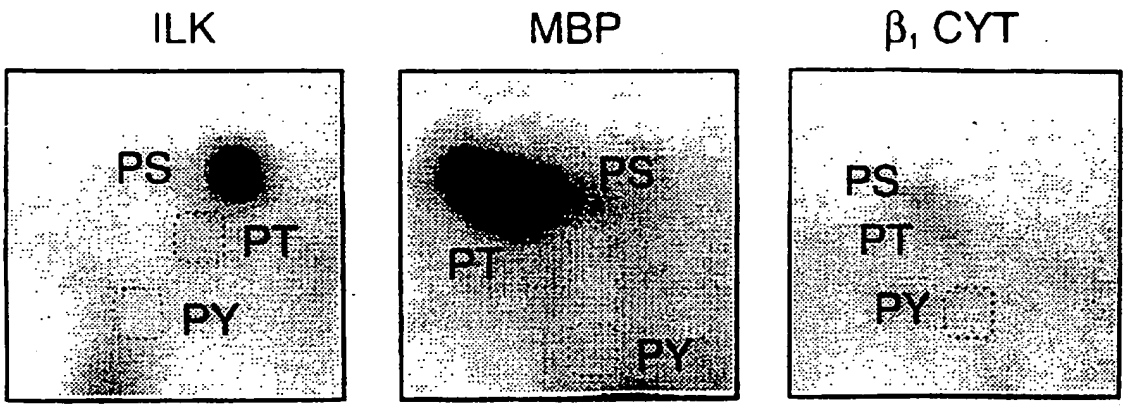

Fig. 2c

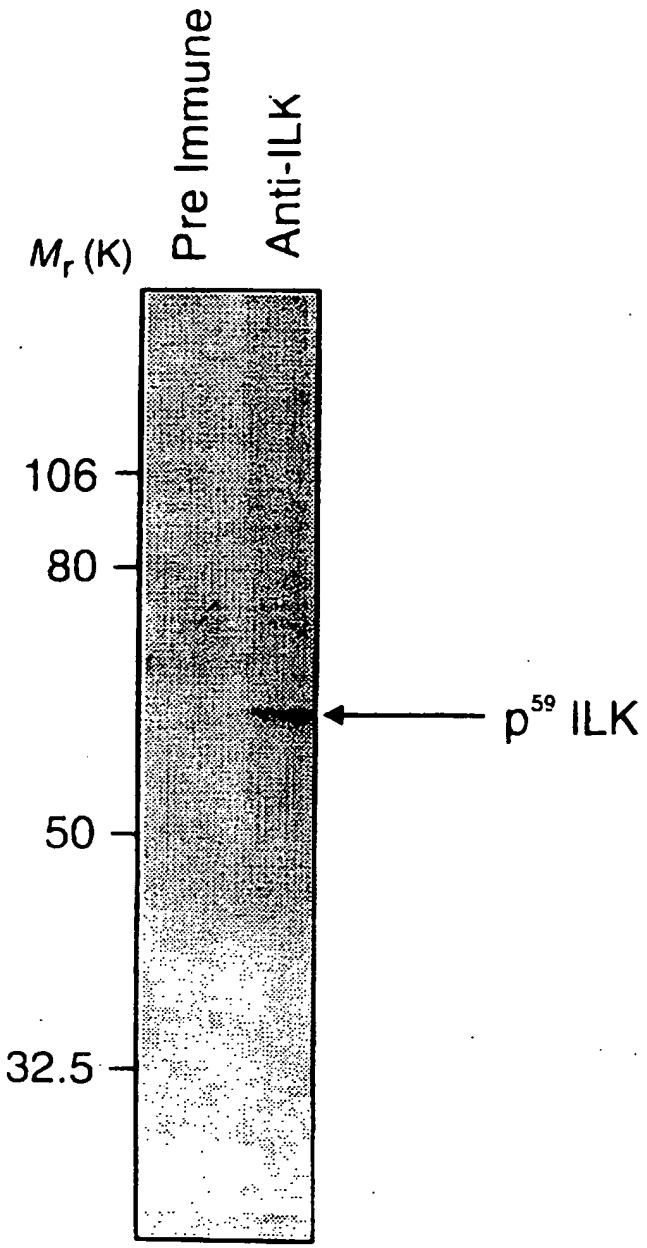

Fig. 3a

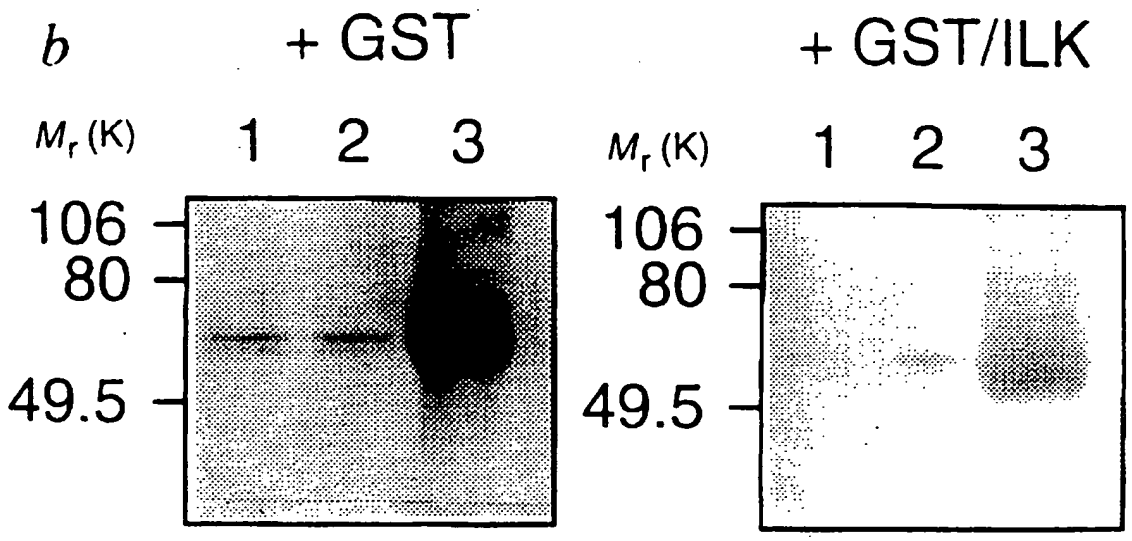

Fig. 3b

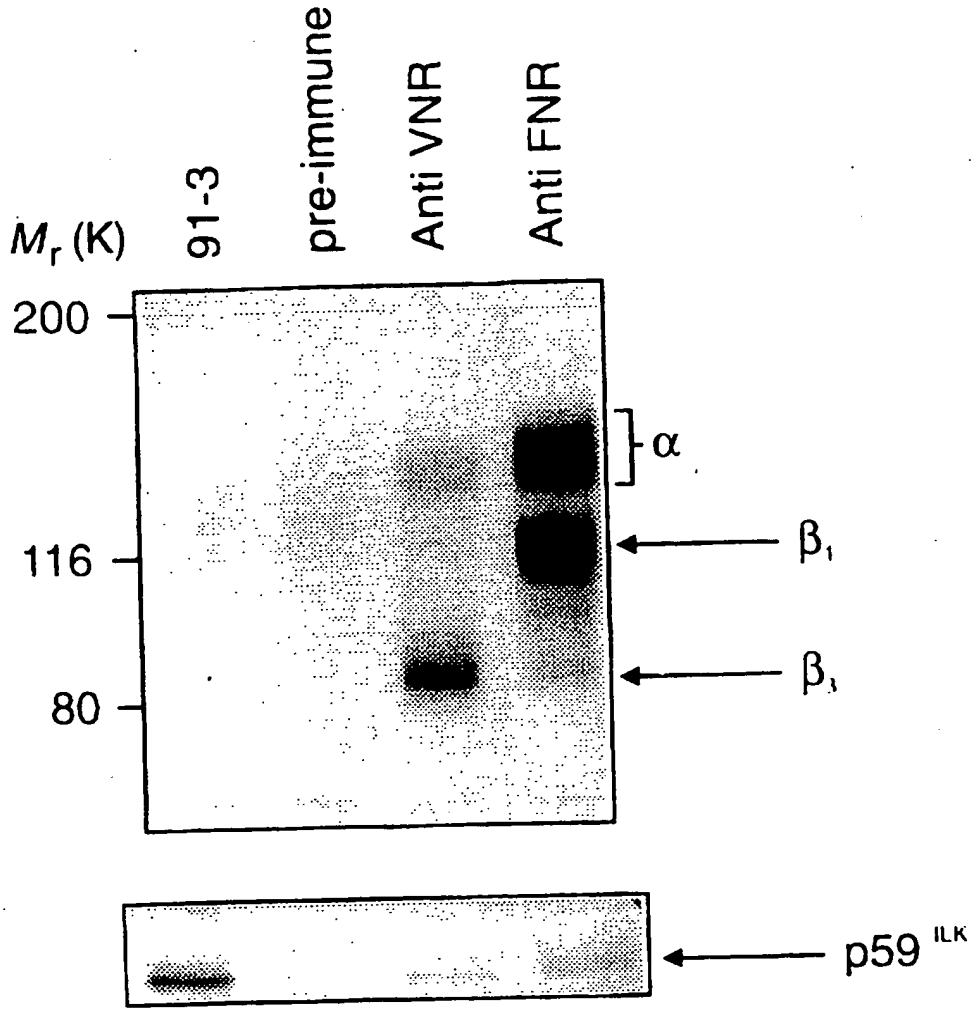
91-3
pre-immune
Anti VNR
Anti FNR
M_r (K)
200
116
80
α
β_1
β_3
p59 ILK

Fig. 3c

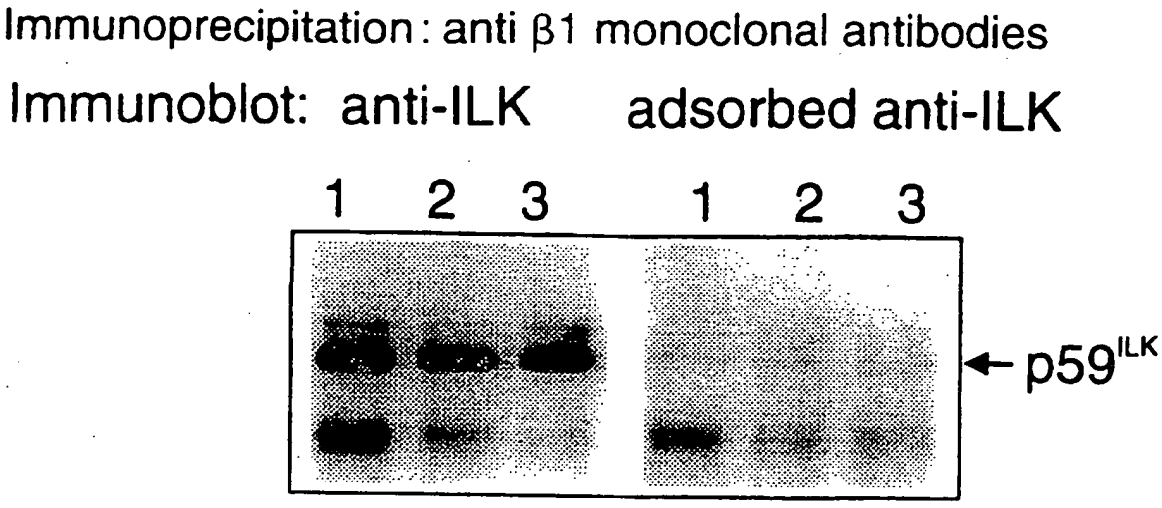
Immunoprecipitation : anti β1 monoclonal antibodies
Immunoblot: anti-ILK adsorbed anti-ILK
1 2 3 1 2 3
p59ILK

Fig. 3d

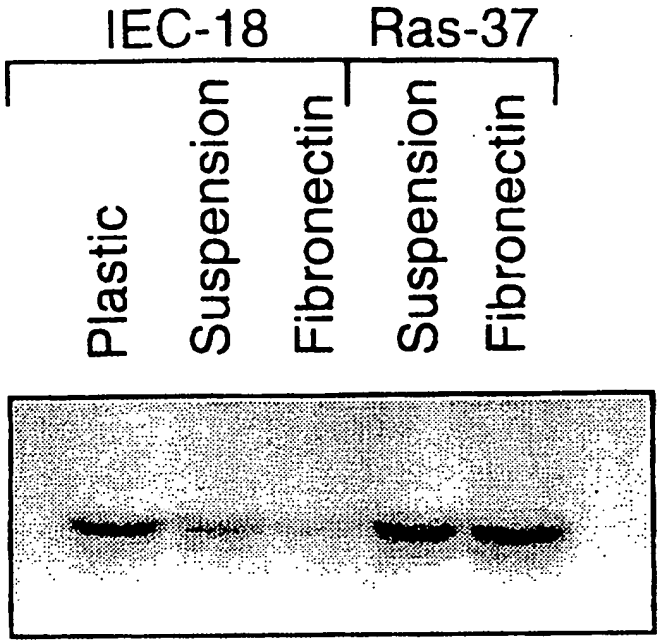
IEC-18
Ras-37
Plastic
Suspension
Fibronectin
Suspension
Fibronectin

Fig. 4a

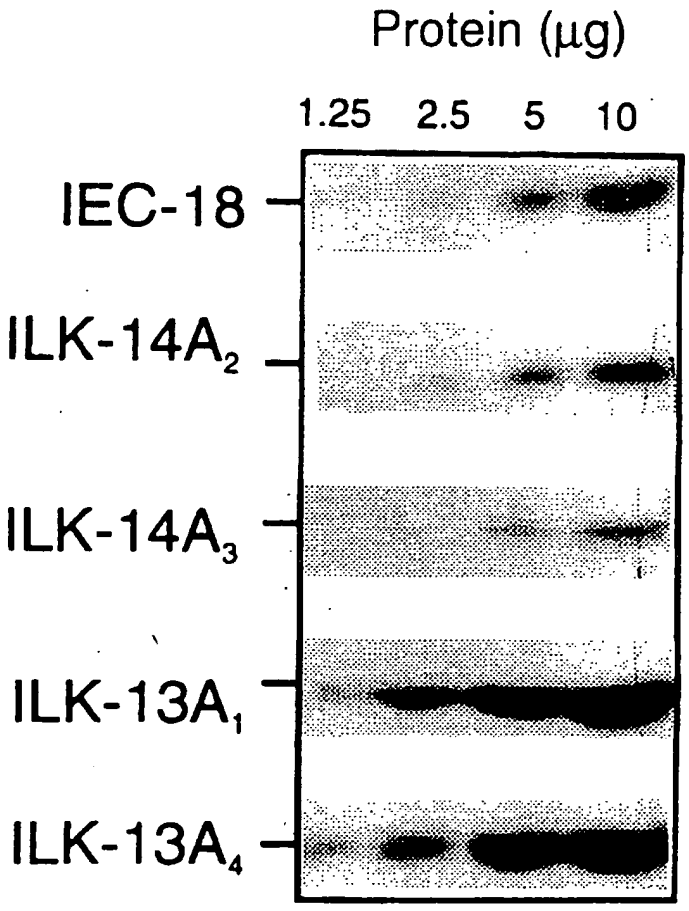
Protein (µg)
1.25
2.5
5
10
IEC-18
ILK-14A2
ILK-14A3
ILK-13A1
ILK-13A4

Fig. 4b

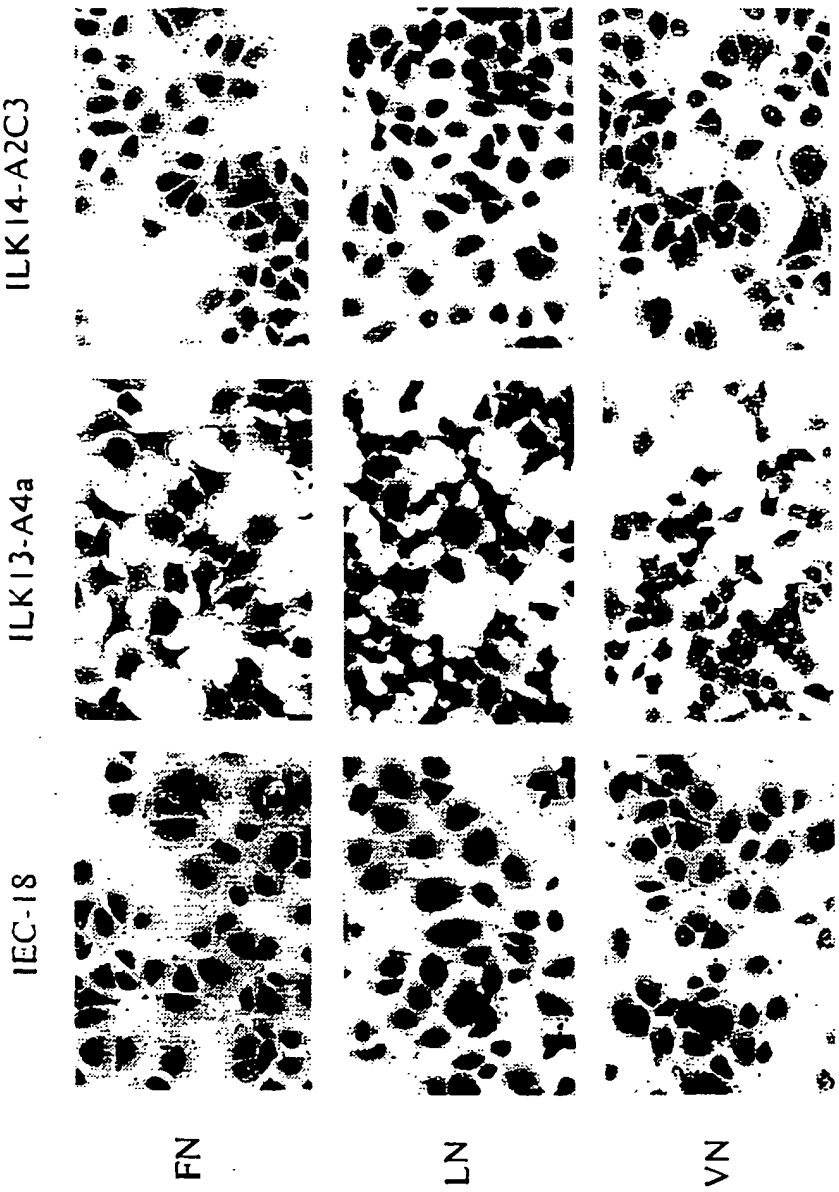

Fig. 4c

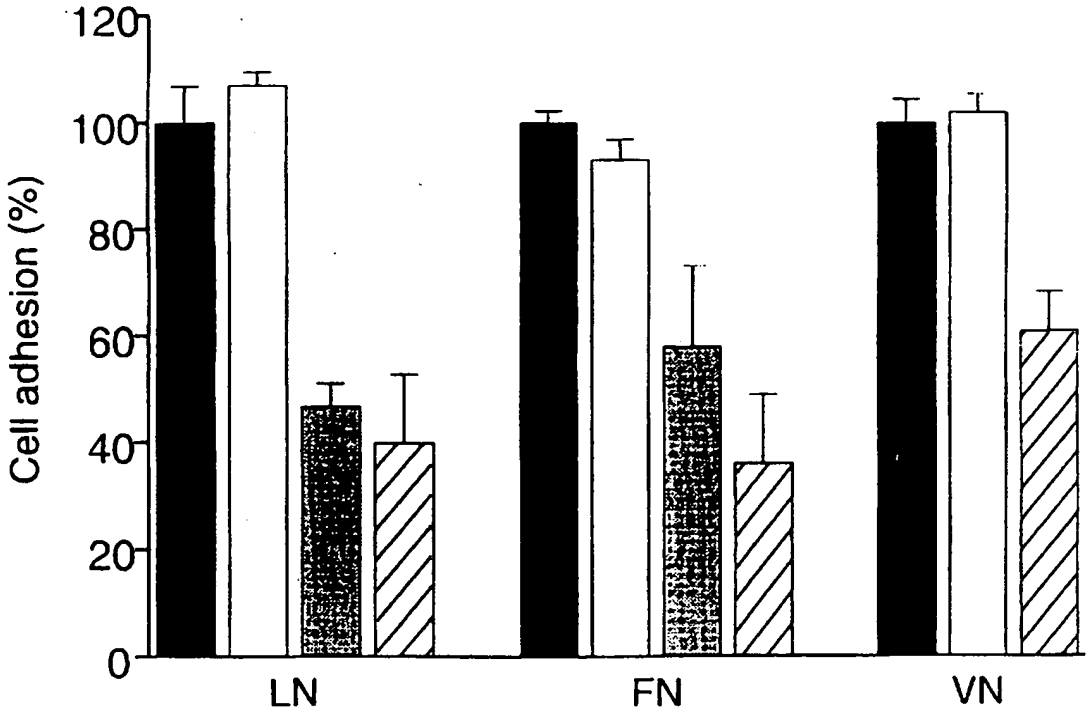

Fig. 4d

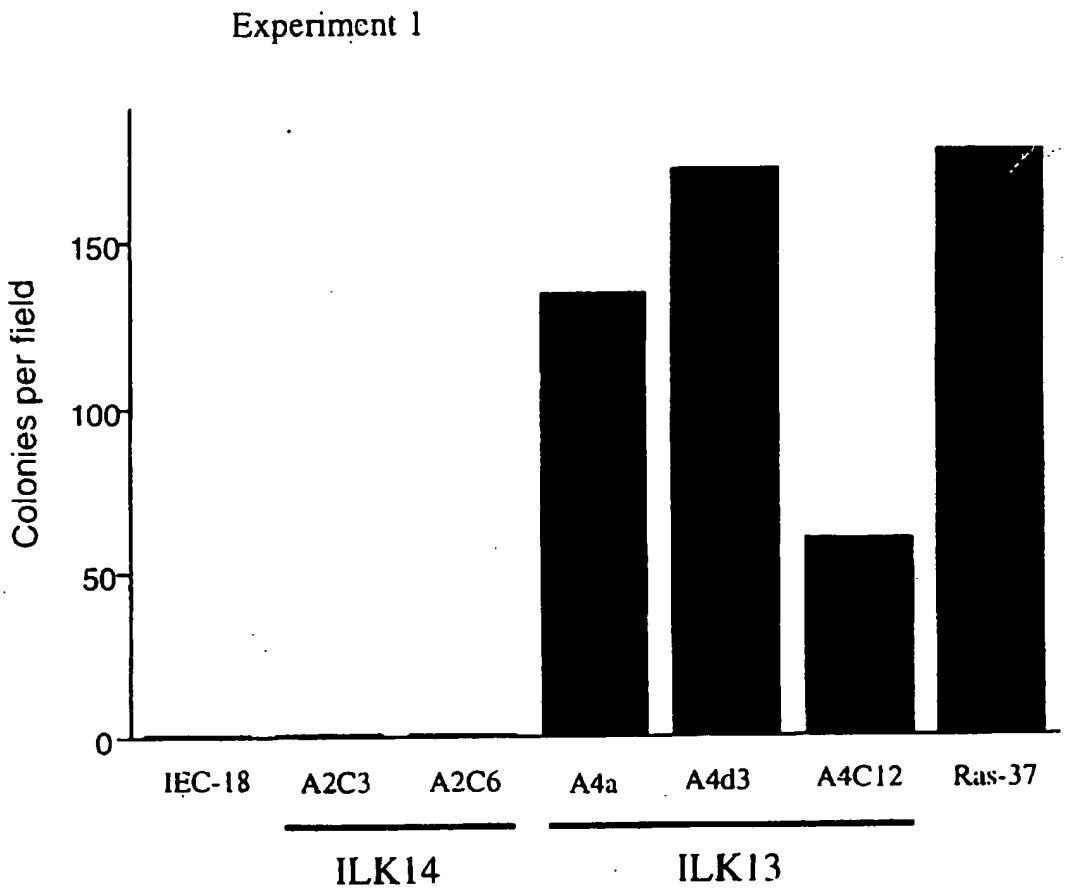

Fig. 4e (continued on page 21/23)

SUBSTITUTE SHEET (RULE 26)

Experiment 2

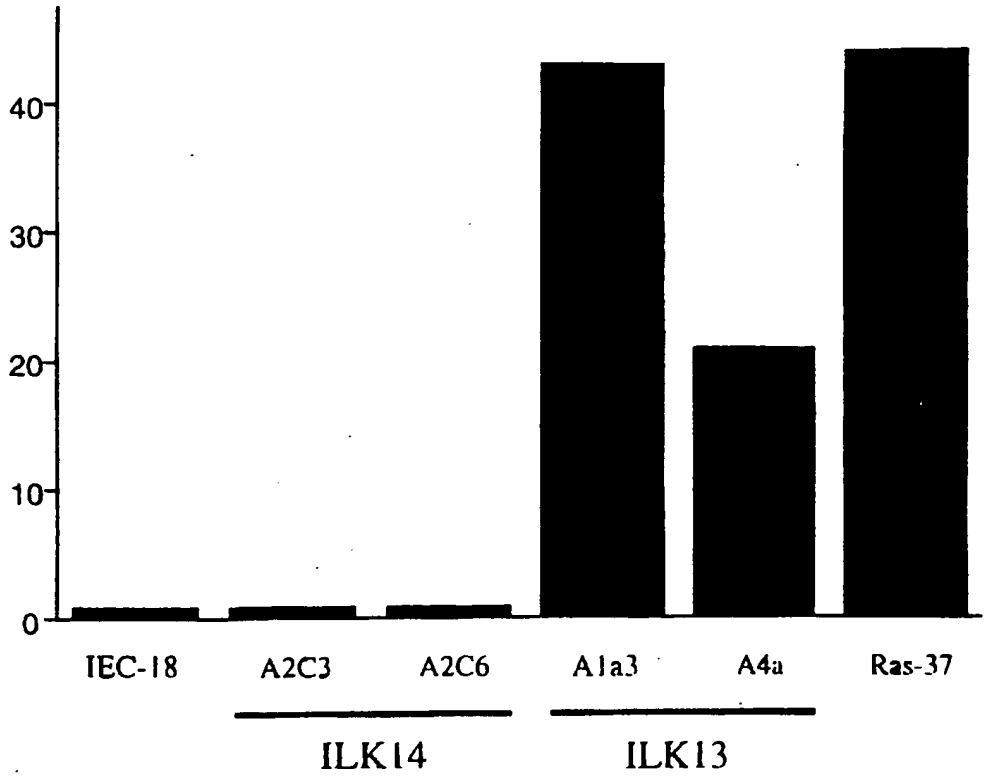

Fig. 4e

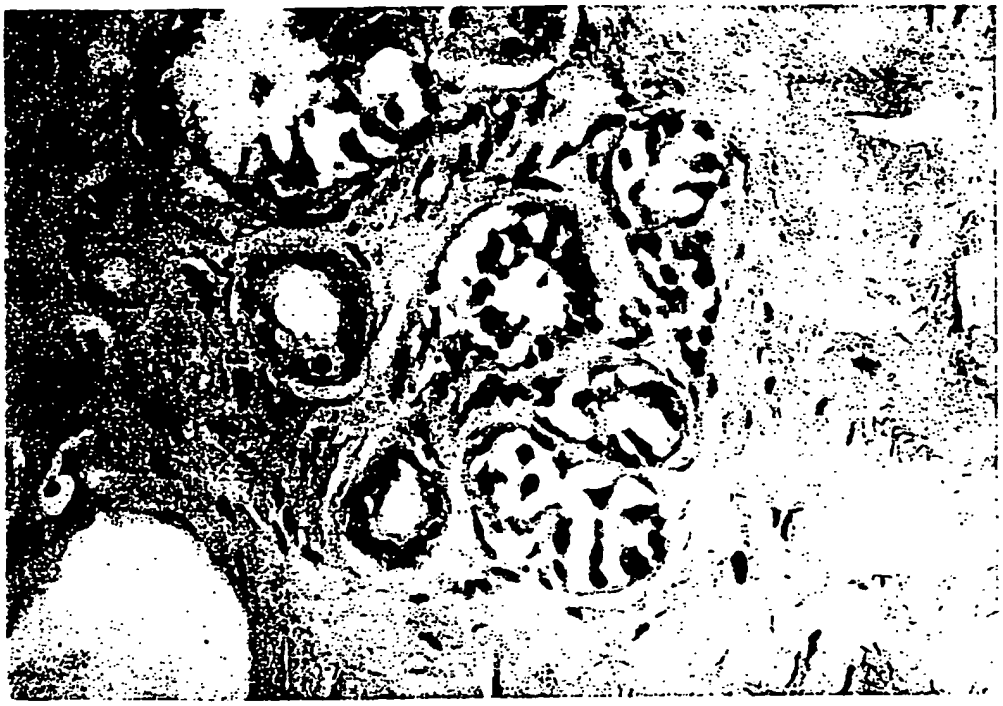

Fig. 5a

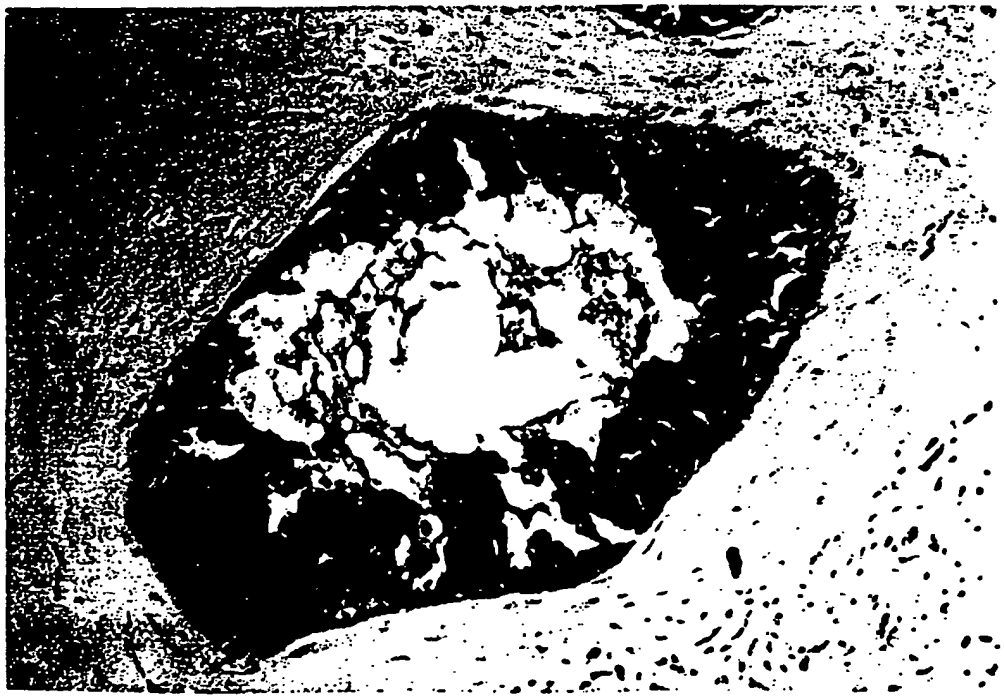

Fig. 5b

Fig. 5c

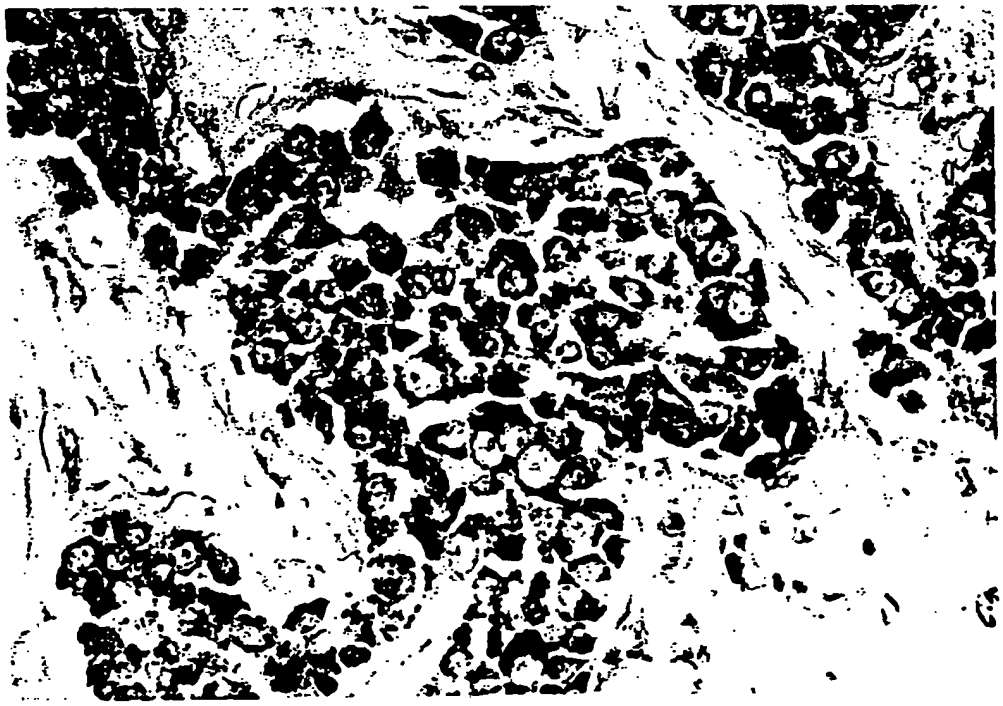

Fig. 5d

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 96/00760

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/52 C12N9/12 A61K38/48 A61K31/70 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	86TH ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, March 1995, TORONTO, ONTARIO, CANADA, page 361 XP000673939 HANNIGAN, G.E., ET AL.: "CLONING OF A NOVEL PROTEIN KINASE ASSOCIATED WITH BETA1-INTEGRIN CYTOPLASMIC TAILS" see the whole document ---	1-4
X	MOLECULAR BIOLOGY OF THE CELL, vol. 6, no. SUPPL., November 1995, page 2244 XP000673935 HANNIGAN,G.E., ET AL.: "OVEREXPRESSION OF A NOVEL INTEGRIN LINKED KINASE (ILK) INDUCES A TRANSFORMED PHENOTYPE AND CYCLIN D1 EXPRESSION" see the whole document ---	1-4

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 May 1997	03.06.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Holtorf, S

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INTERNATIONAL SEARCH REPORT

In' tional Application No
PCT/CA 96/00760

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 1, 6 January 1995, pages 269-273, XP002031634 MORINO, N., ETAL . : "MATRIX/INTEGRIN INTERACTION ACTIVATES THE MITOGEN-ACTIVATED PROTEIN KINASE, p44erk-1 AND p42erk-2" see the whole document	1,2
X	EMBL SEQUENCE DATA LIBRARY, 25 October 1995, HEIDELBERG, GERMANY, XP002031635 HILLIER, L. , ET AL.: "THE WASHU-MERCK EST PROJECT" ACCESSION No. H70160	3,4
X	WO 94 21781 A (MAX PLANCK GESELLSCHAFT) 29 September 1994 see the whole document	11,15
X	EP 0 616 032 A (MITSUBISHI CHEM IND) 21 September 1994 see the whole document	13-15
P,X	CURRENT OPINION IN CELL BIOLOGY, vol. 8, no. 5, October 1996, pages 657-669, XP000673950 DEDHAR,S. AND HANNIGAN,G.E.: "INTEGRIN CYTOPLASMIC INTERACTIONS AND BIDIRECTIONAL TRANSMEMBRANE SIGNALLING" see the whole document	1
P,X	NATURE, vol. 379, 4 January 1996, pages 91-96, XP002031636 HANNIGAN, G.E., ET AL . : "REGULATION OF CELL ADHESION AND ANCHORAGE-DEPENDENT GROWTH BY A NEW BETA1-INTEGRIN-LINKED PROTEIN KINASE" see the whole document	1-4
E	WO 96 36642 A (DAVIS ROGER J ;GUPTA SHASHI (US); RAINGEAUD JOEL (FR); DERIJARD BE) 21 November 1996 see the whole document	13-15,17

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA 96/00760

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 11-17
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 11-17 (as far as in vivo methods are concerned) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/ composition.

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Remark: Claim 16 was read as referring to claim 11; the claims 15 and 17 accordingly referring to the preceding claims 11,12,13 and 14.

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

In tional Application No
PCT/CA 96/00760

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9421781 A	29-09-94	US 5459036 A	17-10-95
		AU 6511994 A	11-10-94
		CA 2158544 A	29-09-94
		EP 0689588 A	03-01-96
		JP 8510897 T	19-11-96
EP 0616032 A	21-09-94	JP 6253835 A	13-09-94
		JP 6329551 A	29-11-94
		CA 2116460 A	03-09-94
WO 9636642 A	21-11-96	AU 4904696 A	29-11-96

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